Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions
VIII. Pathological consequences of rotavirus infection and its enterotoxin

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Morris, Andrew P., and Mary K. Estes. Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions. VIII. Pathological consequences of rotavirus infection and its enterotoxin. Am J Physiol Gastrointest Liver Physiol 281: G303–G310, 2001.—Rotaviral infection in neonatal animals and young children leads to acute self-limiting diarrhea, but infected adults are mainly asymptomatic. Recently, significant in-roads have been made into our understanding of this disease: both viral infection and virally manufactured nonstructural protein (NSP)4 evoke intracellular Ca2+ ([Ca2+]i) mobilization in native and transformed gastrointestinal epithelial cells. In neonatal mouse pup mucosa models, [Ca2+]i elevation leads to age-dependent halide ion movement across the plasma membrane, transepithelial Cl− secretion, and, unlike many microbial enterotoxins, initial cyclic nucleotide independence to secretory diarrhea. Similarities between rotavirus infection and NSP4 function suggest that NSP4 is responsible for these enterotoxigenic effects. NSP4-mediated [Ca2+]i mobilization may further facilitate diarrhea by signaling through other Ca2+-sensitive cellular processes (cation channels, ion and solute transporters) to potentiate fluid secretion while curtailing fluid absorption. Apart from these direct actions in the mucosa at the onset of diarrhea, innate host-mediated defense mechanisms, triggered by either or both viral replication and NSP4-induced [Ca2+]i mobilization, sustain the diarrheal response. This secondary component appears to involve the enteric nervous system and may be cyclic nucleotide dependent. Both phases of diarrhea occur in the absence of significant inflammation. Thus age-dependent rotaviral disease represents an excellent experimental paradigm for understanding a noninflammatory diarrhea.

ROTAVIRAL DIARRHEAL ILLNESS is one of the most common infectious diseases in preschool children. Approximately 3 million children worldwide die of diarrhea annually, of which 600,000–800,000 deaths are attributed to rotavirus (RV). Most of these deaths occur in developing countries (the Indian subcontinent, sub-Saharan Africa, and some areas of Central and South America), where a child’s risk of mortality after infection is estimated to be 1 in 200 or greater. The first licensed vaccine significantly prevented severe disease [1 million children vaccinated, 69–91%] but was voluntarily withdrawn from the United States, and thence the world market, because of a significantly increased relative risk of intussusception after the first or second dose of the vaccine (22). The continuing need for new therapeutic approaches for RV disease prevention and treatment highlights the great necessity for better understanding of the pathobiology underlying this disease. This themes article focuses on new and interesting developments in this area. The reader is also referred to several reviews relevant to rotavirus infection (5, 28). Limitations on the number of citations allowed in this article format have necessarily meant that many important papers have not been credited.

PATHOPHYSIOLOGY OF ROTAVIRAL GASTROENTERITIS

Individuals from all age groups are susceptible to RV infection, but diarrhea is predominantly induced in young children, in whom infections are the most frequent source of acute, self-limiting diarrheal disease at the ages of 6 months to 1–2 years. Thus a clear age dependence exists. RV is relatively unique in this respect, because many bacterial toxigenic diarrheas cause severe clinical disease in children but exhibit no age dependence or predominantly affect older age groups (i.e., the highest incidences of most Vibrio chol-
era and *Escherichia coli* toxigenic diarrheas occur in adolescent/adult populations; Ref. 9). Besides diarrhea, other clinical features of RV disease include anorexia, depression, dehydration, and vomiting. In developing nations, death attributed to severe dehydration secondary to gastrointestinal fluid loss is often exacerbated by the poor nutritional status of these children and high incidences of concomitant infection with other gastrointestinal pathogens (5, 9).

RV has been shown to chronically infect immunocompromised children within the 6–18-month age group and older children and adults. Under these circumstances, sporadic longer-term diarrheal disease is recorded and has been associated with a prolonged period of viral replication and shedding. Normally, both viral replication and shedding resolve within 5–12 days of the onset of infection and gastrointestinal inflammation is low and infrequent or absent. RV-induced diarrhea in older populations can be explained by age-dependent decreases in immune status and innate defense. This diarrhea is correspondingly more complex than that recorded in infants (9). Our understanding in this area is accordingly very limited. What do we know concerning the mucosal and cellular mechanisms underlying noninflammatory RV diarrhea in children? Recent research from a number of different areas has expanded our knowledge.

**VIROLOGICAL FINDINGS**

Considerable effort has been expended in identifying viral proteins causally linked to diarrhea. Studies have clearly shown that, although the onset of watery diarrhea follows detection of virus particles in the stool and intestinal contents (usually within 12 h after infection), viral shedding may continue days to weeks after symptomatic recovery, depending on immunocompetence in both humans and animal models (6). Thus, although viral infectivity is important for disease initiation, there is a poor correlation thereafter. The search for causative viral genes and proteins through genetic reassortment studies of this double-stranded RNA virus (containing 11 genes) has identified a number of virulence factors: genes that encode structural proteins (VP3, VP4, VP6, and VP7) and genes that encode nonstructural proteins [NSP1, NSP2, and NSP4; reviewed in Ref. 8]. VP4 and VP7 are capsid proteins found on the outer proteinaceous layer of the virus. VP4 is important for viral adsorption and penetration into epithelial cells, and VP7 may also play a role in these functions. VP3 and VP6 encode proteins required for RNA transcription and correct viral structure. Little is understood concerning the functions of most of the nonstructural proteins; they may facilitate viral replication and thus increase the efficiency of virus formation. However, NSP4 is the first described viral enterotoxin. NSP4 has uniquely been shown to promote Ca^{2+}-mediated enterotoxigenic effects causally linked to diarrhea. Although there is little evidence for the direct roles of any other RV proteins in mediating enterotoxigenic diarrhea, their requirement in the replication process for efficient viral production suggests that they may indirectly influence late stages of diarrhea when the buildup of cellular viral proteins appears to trigger nonimmune innate host-defense mechanisms that sustain and potentiate the mucosal enterotoxigenic effects of NSP4.

**USE OF ANIMAL MODELS**

Because of the impossibility of studying RV disease in children or surgically excised human neonatal tissues for ethical reasons, most of our current understanding of this multifactorial disease comes from studies in animal models. Both large and small mammals exhibit RV infection and pathogenesis (reviewed in Ref. 6). Disease severity and location within the gastrointestinal tract vary among animal species, inoculum used (viral strain and dose), immune status, age on infection, and host intestinal physiology. Age-dependent RV disease is a major agricultural concern for large-animal (calves, foals, lambs, and piglets) breeding facilities. However, studies in these models are prohibitively expensive. As a result, the best-characterized model is presently the mouse, with investigations in both rabbit and rat models also being conducted (5). Naive mice challenged with low doses of murine RV become infected (homologous infection) and exhibit age-dependent diarrheal symptoms. Furthermore, infection spreads to other naïve mice with equal incidence in both pup and adult populations in terms of the intensity and duration of virus shedding. Similar phenomena are recorded for all other homologous and heterologous (e.g., nonmurine virus infection in mice) virus-mammalian host-specific interactions. Viral clearance is linked to the development of virus-specific intestinal IgA, similar to human infection (12). Furthermore, age-dependent resistance to RV infection appears to be mediated by acquired immunity, and a similar mechanism is thought to be operative as children age.

The mucosal site of rotavirus interaction in animal models, as in humans, is usually limited to mature enterocytes at the tips of the villi in the small intestine. However, segmental variability (duodenum > jejunum > ileum) can exist between animal species and between homologous and heterologous RV infection. It is not known whether these tropisms reflect the restricted location of a specific receptor for cellular viral entry or whether differentiated enterocytes express other factors required for efficient infection and replication. The identification of a cellular receptor for human RV, and its homologues in animal models, will clarify these issues.

**PHYSIOLOGICAL FINDINGS**

A number of reviews have outlined hypotheses explaining age-dependent RV diarrhea in young children and animals (5, 8). Watery diarrhea may be caused by 1) changes in small intestinal surface area, leading to a reduction in net fluid absorption at a time when the colonic absorptive reserve may not be fully developed, 2) changes in mucosal osmotic permeability secondary to mucosal destruction, and 3) changes in fluid and
Direct Virus-Mediated Diarrhea: Cellular Effects of Rotavirus NSP4 Enterotoxin

Rotavirus infection has been shown by a number of groups to cause sustained intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) mobilization in gastrointestinal cell lines (28), and the pleiotropic consequences of elevated [Ca\(^{2+}\)]\(_i\) have led to the generation of several new ideas (Fig. 1).

The first of these relates to the fact that rotavirus infection and elevated [Ca\(^{2+}\)]\(_i\) in cultured, nonpolarized epithelial cells leads to cytolysis. It has therefore been hypothesized that similar events occur in vivo, leading to a loss of small intestinal surface mucosa and reduced fluid absorptive area. This cellular destruction scenario would explain the dramatic villus flattening observed in pig and calf models. However, such dramatic histological changes would also be expected to be associated with activation of significant immune responses, similar to those reported for mucosal-damaging bacterial toxins elaborated during dysentery and invasive diarrheal syndromes. This would result in a large inflammatory component to diarrhea. However, this does not occur in children and in many animal models. In mice, rotavirus infection is not associated significantly with mucosal inflammation; only mild mononuclear infiltration of the lamina propria is seen (4, 8, 12), and histopathological changes are limited to cytoplasmic vacuolization in a percentage of small intestinal enterocytes at the tips of the villi. Pigs, rabbits, calves, and lambs exhibit more pronounced histological changes that are seen at the site of viral replication 24–72 h after infection. The conclusion that mucosal destruction is unlikely to play a primary role in the propagation of diarrhea in animals is based on the findings that 1) diarrheal onset occurs during subclinical levels of infectious load, before alterations in cytopathology, 2) prophylactic treatment with cytoprotective growth factors inhibits histologic changes in pig models while failing to affect diarrhea (reviewed in Ref. 8), and 3) polarized monolayers of cultured epithelial

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**Fig. 1.** The pleiotropic cellular consequences of rotavirus (RV)- and NSP4-elicited intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) mobilization. A growing list of Ca\(^{2+}\)-dependent processes that are altered in a negative (-) or positive (+) manner by [Ca\(^{2+}\)]\(_i\), mobilization include downregulation of enzymes and hydrolases involved in Na\(^+\)-hexose absorption, changes in junctional integrity, actin-dependent cell contact, and constitutive vesicle trafficking within the cellular biosynthetic pathway. Sustained [Ca\(^{2+}\)]\(_i\) mobilization, together with the build-up of viral proteins within the cell, is expected to affect native protein folding and to activate a variety of local nuclear factor-B-dependent cellular processes, explaining changes in secreted cytokine profiles observed after viral infection that are unlikely to contribute directly to secretory diarrhea. Not surprisingly, many of the positive effects seen inside the cell stimulate viral synthesis and release. Further characterization of both types of phenomena is required.

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**WHAT CELLULAR MECHANISMS DEFINE RV DIARRHEA?**

There are both cellular and clinical definitions of secretory diarrhea. The former is based on observations made in vitro and states that epithelial cells exhibit complex but coordinated interactions among plasma membrane channels, pumps, and cotransporters to cause electrogenic exit of Cl\(^-\) across the apical plasma membrane. The accompanying paracellular movement of Na\(^+\) and H\(_2\)O leads to accumulation of fluid within the lumen of the gastrointestinal tract and, subsequently, diarrhea. Clinically, secretory diarrhea is diagnosed when measurements of fecal fluid electrolyte secretion. All may contribute at different times to diarrheal production.

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**DIRECT VIRUS-MEDIATED DIARRHEA: CELLULAR EFFECTS OF ROTAVIRUS NSP4 ENTEROTOXIN**

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**Fig. 1.** The pleiotropic cellular consequences of rotavirus (RV)- and NSP4-elicited intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) mobilization. A growing list of Ca\(^{2+}\)-dependent processes that are altered in a negative (-) or positive (+) manner by [Ca\(^{2+}\)]\(_i\), mobilization include downregulation of enzymes and hydrolases involved in Na\(^+\)-hexose absorption, changes in junctional integrity, actin-dependent cell contact, and constitutive vesicle trafficking within the cellular biosynthetic pathway. Sustained [Ca\(^{2+}\)]\(_i\) mobilization, together with the buildup of viral proteins within the cell, is expected to affect native protein folding and to activate a variety of local nuclear factor-B-dependent cellular processes, explaining changes in secreted cytokine profiles observed after viral infection that are unlikely to contribute directly to secretory diarrhea. Not surprisingly, many of the positive effects seen inside the cell stimulate viral synthesis and release. Further characterization of both types of phenomena is required.
The novel discovery of a rotavirus-produced enterotoxin was made by serendipity when individual RV genes were expressed in cells (Ref. 8; Fig. 2A). The nonstructural protein NSP4 was identified as the only gene product capable of eliciting \([Ca^{2+}]_i\) mobilization, thus mimicking RV infection. Furthermore, when mice were injected with a 22-amino acid peptide synthesized from the COOH terminus of this 175-amino acid protein, diarrhea was recorded within 4 h. The full-length protein similarly produced diarrhea, whereas peptides from regions outside of amino acids 96–135 did not. Diarrhea mimicked that recorded for virulent, homologous virus infection, exhibiting a similar severity but less prolonged time course. Furthermore, immunization of pregnant dams with physiologically active NSP4<sub>114–135</sub> peptide conferred resistance against homologous RV-elicited diarrhea to pups, confirming a major role of this peptide in the pathogenic process. When NSP4<sub>114–135</sub> was added to pup small intestinal mucosal sheets mounted in Ussing chambers, a \(Ca^{2+}\)-dependent component to transepithelial Cl<sup>-</sup> secretion was recorded. However, whereas this current was macroscopically similar to carbachol responses in pup mucosa, it was lost in adult mucosa. A unique age dependency similar to the whole animal diarrheal response was therefore demonstrated for NSP4 and not for any other RV protein.

More recently, in vitro studies have shown that after viral replication in cells, a 7-kDa peptide of NSP4 containing a physiologically active COOH-terminal domain (amino acids 112–175) is released into the medium of virus-infected cells via a nonclassic, Golgi-independent cellular secretory pathway (33). This suggests that extracellular NSP4 (eNSP4) is available for subsequent enterotoxigenic effects on the surrounding mucosa and provides a physiological basis for immunoprotection after NSP4 immunization. As indicated by recombinant protein studies, this endogenously produced eNSP4 peptide binds to an as yet unidentified apical membrane receptor to mobilize \([Ca^{2+}]_i\) through phospholipase C (PLC) signaling and to preferentially stimulate \([Ca^{2+}]_i\)-sensitive halide influx into neonatal, but not adult, intestinal enterocytes and colonocytes. The fact that \([Ca^{2+}]_i\) mobilization occurs regardless of mucosal age but differs from classic PLC agonists in its pertussis toxin sensitivity (unpublished observation) and age-dependent secretory activity supports the hypothesis that NSP4's enterotoxic actions occur distal to the \(Ca^{2+}\) rise, at the level of the plasma membrane anion movement. NSP4 is proposed to elicit anion secretion through an interaction with luminal, age-dependent \(Ca^{2+}\)-sensitive anion channels.

Secondary effects on basolateral \(Ca^{2+}\)-sensitive \(K^+\) conductances, which could facilitate transcellular Cl<sup>-</sup> secretion via cellular hyperpolarization and the up-regulation of basolateral secondary active Cl<sup>-</sup> uptake (20), together provide a cellular basis for the age-dependent transcellular secretion recorded during RV diarrhea. Evidence supporting this hypothesis comes from studies made in cystic fibrosis (CF) transmembrane conductance regulator (CFTR)-deficient mice (21). In CFTR-deficient CF mouse pups, NSP4 continues to evoke both age-dependent diarrhea and age-dependent \([Ca^{2+}]_i\)-sensitive changes in enterocyte and colonocyte plasma membrane halide influx. In contrast, the classic \([Ca^{2+}]_i\)-mobilizing secretagogue carbachol and the cAMP-mobilizing diterpene forskolin fail to provoke similar phenomena. Thus RV-induced secretory diarrhea is not mediated by CFTR, and the molecular identity of the responsible channel remains to be determined.

This unique aspect of NSP4-mediated \([Ca^{2+}]_i\) signaling (i.e., a role as the first described viral enterotoxin) may represent only part of the overall picture. Dramatic intracellular NSP4 production recorded 24 h after RV infection, as evidenced by in situ hybridization studies in intestinal villi isolated from virus-infected cells, in contrast to their nonpolarized counterparts, fail to exhibit cytolysis when infected with RV (15).

**Fig. 2.** Comparison of the known effects of viral NSP4 enterotoxin and RV on net mucosal fluid production. A: only direct mucosal effects have so far been investigated for extracellular NSP4 enterotoxin (eNSP4), which mimic those reported for viral infection with the subtle difference that virally induced disease is more prolonged. NSP4 enterotoxin may also be involved in second-stage enteric nervous system (ENS)-mediated disease, but this has yet to be tested. B: RV infection appears to evoke both mucosal cell (primary) and ENS (secondary) effects on mucosal fluid loss. Mucosal cell responses are linked to elevations of \([Ca^{2+}]_i\), and distal, \(Ca^{2+}\)-sensitive, age-dependent changes in cellular plasma membrane anion movement and transmucosal anion secretion. Both are cyclic nucleotide independent. During later stages of the disease, incipient reactive hyperemia is associated with secondary ENS effects proposed to prolong and potentiate the primary NSP4 signal. These may be cyclic nucleotide dependent.
sorptive sugar transport (reviewed in Ref. 14). Recent plain decreases in apical hydrolase polarization indices and protein folding (reviewed in Ref. 1) may ex-
and G protein-sensitive intracellular vesicular trans-
ing of a variety of host cell Ca \(^{2+}\) may underlie the initial ischemic re-
ance. This innate defense mechanism also appears to be active in a number of other conditions including intest-
 hypersensitivity reactions in antigen-challenged rodents, after mechanical stimulation of the mucosa, in small intestinal arterial blood supply in many animal models including rodents. In the latter, ultrastructural changes similar to RV are first seen at the tip of the villi, the extent of which determines the severity of clinical illness (31). However, if occlusion is re-
etransplantation during this period may underlie many cellular responses recorded after RV infection. When coupled with virus-mediated repression of endogenous (host cell) protein synthesis, [Ca\(^{2+}\)], mobilization is expected to affect the functioning of a variety of host cell Ca\(^{2+}\)-sensitive processes, enzymes, and transporters. Thus inhibition of Ca\(^{2+}\)-
and G protein-sensitive intracellular vesicular transport and protein folding (reviewed in Ref. 1) may explain decreases in apical hydrolase polarization indices recorded during RV infection (15) and changes in absorptive sugar transport (reviewed in Ref. 14). Recent evidence additionally suggests that NSP4 may directly inhibit the functioning of the cellular Na\(^{+}\)-dependent glucose transporter SGLT-1 (14). Extracellular and/or intracellular NSP4 may further contribute to diarrheal pathogenesis by altering the dynamics of intracellular actin distribution and intracellular contacts, as well as effecting changes in paracellular permeability (reviewed in Ref. 5). All of the above phenomena are evident after viral replication.

Another related and potentially important new finding has been the publication of the crystal structure for NSP4(495–137) (3). Modeling predicts that this portion of the full-length NSP4 molecule, encompassing the enterotoxigenic peptide sequence (NSP4(114–135)), may form a homotetrameric pore. This could potentially span the cellular endoplasmic reticulum (ER) membrane and act as a Ca\(^{2+}\) channel. No direct evidence currently supports this hypothesis. However, 1) the interior hydrophilic surface of the predicted pore contains a metal (Ca\(^{2+}\))-binding region (3 and 2) previous studies (reviewed in Ref. 8) have shown that endog-
enously expressed NSP4, although failing to alter plasma membrane divalent cation permeability, can induce non-PLC-dependent changes in ER membrane Ca\(^{2+}\) release when expressed within cells. This poten-
tially important secondary mode of NSP4-mediated [Ca\(^{2+}\)]; mobilization, again requiring intracellular NSP4 synthesis, may both potenti-ate the enterotox-
genic [Ca\(^{2+}\)]; signal of extracellular NSP4 or itself initiate disease at later times after infection.

SECONDARY HOST-MEDIATED DIARRHEA: VILLUS ISCHEMIA AND INVOLVEMENT OF THE ENTERIC NERVOUS SYSTEM

RV infection in mouse pups has been correlated with changes in vascular circulation and reversible cellular ischemia in the villus tip during the periods of highest viral replication [48–72 h after infection (24); Fig. 2B]. After this period, incipient hyperemia is recorded for another 24–48 h before a second phase of ischemia leading to the initiation of villus damage. The signifi-
cance of these detailed ultrastructural studies has un-
til recently been difficult to ascertain. The original authors suggested that release of a vasoactive subst-
ance was likely to underlie the initial ischemic re-
sponse, leading to functional changes to, but not loss of, mucosal enterocyte integrity. Whether similar phe-
omena are widespread in other RV-infected animal models has not been clearly researched. However, other microbial toxins, most notably choleragen toxoid, have been shown to cause parallel and often exagger-
ated structural changes in human small intestinal villi, the extent of which determines the severity of clinical illness (18). Furthermore, the duration of both cholera and RV diarrhea illness is reduced after oral aspirin (nonsteroidal anti-inflammatory) therapy, and elevated levels of prostaglandins (notably PGE\(_2\) and PGF\(_{2\alpha}\)) have been recorded in the plasma and stool of RV-infected children (32) and cholera-infected adults (reviewed in Ref. 17). These facts point toward the existence of similar prostaglandin-dependent cellular mechanisms responsible for ultrastructural changes in both instances. In cholera, this phenomenon is linked to activation of submucosal nervous reflexes that form part of the enteric nervous system (ENS).

Histological staining in rodents and other animal models has clearly demonstrated that vasoactive intestinal polypeptide (VIP) is ubiquitously expressed throughout neuronal cells of the myenteric plexus, establishing it as a major secretagogue released by a number of local ENS intramural reflexes. In cholera, good experimental evidence demonstrates that mucosal enterochromaffin cells release 5-hydroxytrypt-
amine (5-HT). Interactions with mucosal 5-HT\(_2\) receptors and local neuronal 5-HT\(_3\) or 5-HT\(_4\) receptors (species specific) elicit neuronal VIP release. In addi-
tion, mucosal VIP effects and the luminal overflow of mucosally derived PGE\(_2\) (reviewed in Refs. 17 and 25) result in both protein kinase C and phosphoinositol hydrolysis-dependent increases in fluid (Cl\(^{-}\) or HCO\(_{3}\)^{-}) secretion as well as decreases in transmucosal absorp-

This innate defense mechanism also appears to be active in a number of other conditions including intest-
inal hypersensitivity reactions in antigen-challenged rodents, after mechanical stimulation of the mucosa, in diarrheas associated with chemical laxatives and opiate withdrawal, and after acute reversible occlusion of small intestinal arterial blood supply in many animal models including rodents. In the latter, ultrastructural changes similar to RV are first seen at the tip of the villi before enterocytes exhibit signs of irreversible damage and progress toward the base with the continuation of ischemia (31). However, if occlusion is re-
moval of cellular ischemic damage, reactive hyper-
peramia again characterizes the response (11). This vasodilation has likewise been shown to be associated with mucosal endocrine cell 5-HT production, and both lidocaine and TTX-sensitive neuronal VIP release are indomethacin sensitive and characterized by net mucosal fluid loss. Thus disparate conditions resulting in nondamaging villus ischemia evoke a stereotypical local neuronal reflex, causing acute reactive hyperemia and accompanied by alterations in mucosal fluid balance. This innate protective mechanism, which could be triggered as a consequence of RV replication in villus tip mucosal cells, may contribute to a second ENS-dependent phase of diarrhea without the involve-
ment of a mucosal inflammatory response.
Alternatively, RV-induced ischemia could evoke acute changes in cellular nitric oxide (NO) production (reviewed in Ref. 27), with corresponding vascular effects leading to mucosal cell PGE\textsubscript{2} production and cGMP-dependent anion secretion via a local nonadrenergic, noncholinergic branch of the ENS. Mucosal cGMP effects differentiate the pathologies of E. coli. heat labile enterotoxin (LT) and heat stable enterotoxin A (STa) from the effects of cholera toxin-stimulated 5-HT production, even though all are dependent on ENS-neuronal reflex pathways (10).

Direct evidence for ENS involvement in late-phase RV-induced diarrhea in mice has been published (16). Treatment of mice with drugs that affect ENS function significantly inhibited RV-mediated net fluid transport in organ bath experiments and altered transmucosal potentials toward values consistent with anion secretion and away from cation absorption. These studies, conducted 48–60 h after infection, were performed in mice exhibiting classic macroscopic signs of reactive hyperemia: tissue edema, vasodilation, and ischemia. Analysis of the data demonstrated that 66% of RV-induced net fluid secretion was mediated by this ENS secretomotor reflex arc because of its sensitivity to TTX (neuronal cation channel inhibitor), lidocaine (local anesthetic), or mesalamine (ganglionic inhibitor). Diarrhea was also inhibited in mice injected intraperitoneally with lidocaine, demonstrating consistency of the effects at the whole animal level.

One clinical consequence of the activation of such a mechanism would be the potentiation but not initiation of diarrhea evoked by NSP4 enterotoxin. In this respect, clinical studies with the enkephalinase inhibitor ragedotril (acetorphan) in hospitalized RV-infected children have shown that this drug reduces diarrhea duration from an average of 48 to 24 h after admission (29). Ragedotril, a useful agent for the treatment of a variety of acute and chronic infectious and inflammatory diarrheas in all age groups, prevents the breakdown of endogenous enkephalins within the gastrointestinal mucosa, notably those that interact with antiserotinergic (cAMP lowering) δ-receptors expressed on mucosal epithelial cells. This prevents elevations in cAMP levels and, hence, cyclic nucleotide-dependent anion secretion. These studies provide strong clinical evidence for the activation of this innate defense mechanism triggered by reactive hyperemia after subtoxic mucosal ischemia. Any subsequent inflammatory burden caused by ischemia-induced mucosal damage, the amplifying effects of local or species-specific proinflammatory cytokine release, or immunodeficiency would then serve to magnify this host-mediated diarrheal component.

**CORRELATIONS AND DIFFERENCES BETWEEN DIARRHEAL EFFECTS OF VIRAL NSP4 AND BACTERIALLY DERIVED TOXINS**

Many infectious agents and all bacterial enterotoxins cause diarrhea by affecting cellular ion secretion. The classic example is the direct mucosal action of cholera toxin, which increases cAMP levels, leading to the opening of the apical plasma membrane cAMP-responsive anion channel to cause fluid secretion. This conductance has been studied extensively (reviewed in Ref. 20) and is a property of CFTR. In CF patients, this channel either does not function very well or does not function at all. This results in a lack of fluid secretion within epithelial organs including the epithelial linings of the gut and airways. Heat-stable enterotoxins produced from both E. coli and *Yersinia enterocolitica* use a parallel pathway to cause secretory diarrhea via elevated cGMP (reviewed in Ref. 23). On the other hand, enteroadherent bacteria such as *Salmonella typhimurium* produce a soluble mediator, flagellin, that potently stimulates an inflammatory diarrhea through nuclear factor (NF)-κB dependent interleukin (IL)-8 production with ultimately the luminal recruitment of polymorphonuclear leukocytes (PMN; Ref. 13). PMN are a source of luminal 5′-AMP that, on conversion to adenosine, leads to receptor activation and CFTR Cl\textsuperscript{−} channel opening. Therefore, a common theme for both enterotoxigenic and enteroadherent bacteria is direct or indirect activation of the cellular CFTR Cl\textsuperscript{−} channel through elevation in cellular cyclic nucleotide levels. Furthermore, these pathways are influenced by the ENS. Noninflammatory and inflammatory conditions cause the release of multiple peptides from the mucosal epithelium and/or luminal PMN, which potentiate this response by stimulating either the cholinergic interneurons or the secretomotor VIPergic afferent nerves within the myenteric plexus to produce the same net hypersecretory effect (reviewed in Ref. 10).

The primary cellular mechanism used by the NSP4 enterotoxin appears to be different from all other enterotoxins. Loss of function of the cAMP-activated CFTR Cl\textsuperscript{−} channel protects individuals with CF and transgenic mice from the diarrheal effects of both *V. cholera* (CTX) and *E. coli* (STa and LT) toxins and, importantly, ENS-VIPergic nerve activation. However, this genotype fails to protect CF children and mice from age-dependent RV diarrhea. Thus age-dependent diarrheal onset induced by RV and NSP4, unlike the non-age-dependent diarrhea recorded after toxigenic increases in cellular cyclic nucleotide levels, does not appear to be mediated by activation of the cellular CFTR Cl\textsuperscript{−} channel. Therapeutic approaches aimed at preventing ENS involvement during RV disease would therefore be expected to affect the second phase of the disease process but not to affect the primary diarrhea initiated by NSP4.

**IS NSP4 ENTEROTOXIN UNIQUE IN ITS MUCOSAL INTERACTIONS?**

The direct mucosal interaction of *E. coli*-derived heat-stable enterotoxin B (STb) may be an exception to the concept that NSP4’s mucosal interactions are unique. Cellular extracts of STb-elaborating *E. coli* have been shown to elicit cyclic nucleotide-independent fluid secretion and to increase short-circuit current across pig small intestinal mucosa mounted in Ussing...
chambers (reviewed in Refs. 7, 26). Measurement of electrolyte content of intestinal segments further suggests that STb stimulates HCO₃⁻ secretion. These changes are associated in vivo with STb-induced vacuolation of villus tip absorptive cells, partial atrophy of the villi, and dilation of intravillus capillary networks without cellular damage or the accompanying signs of inflammation. Previous misunderstandings regarding the apparently unique porcine sensitivity to STb-induced diarrhea, and its lack of effect in other animal models including the mouse, have been addressed by the findings that STb activity could be recorded in other animal models when endogenous luminal protease activity was inhibited.

In mouse intestinal loop assays, purified STb toxin has been confirmed to cause second-phase ENS-mediated diarrhea that is inhibited by aspirin and indomethacin without altering cAMP or cGMP levels; it also causes luminal PGE₂ release and vascular dilation. Furthermore, the quantity of fluid secreted has been correlated with PGE₂ generation and mucosal cell levels of arachidonic and phosphatidic acid metabolism. More recently, both mucosal PGE₂ release and 5-HT generation by intestinal enterochromaffin cells have been shown to underlie this response, which in rodents is partially sensitive to the 5-HT antagonist ketanserin and to drugs that inhibit ENS activity such as lidocaine, tetrodotoxin, and atropine. At the cellular level, STb, like NSP4, has also been shown to cause the mobilization of [Ca²⁺]i in mucosal cell lines through a pertussis toxin-sensitive G protein-dependent mechanism. Thus a number of similarities between NSP4 and STb exist at the level of primary and secondary phases of the disease process.

However, unlike NSP4, STb does not exhibit a pronounced age dependence, and, in fact, disease is nearly always seen in adult animals and is rarely recorded in humans (reviewed in Ref. 7). These results suggest that, whereas NSP4 requires a cellular specificity expressed in neonatal mucosa, STb conversely utilizes a receptor specifically expressed in adult mucosa. Apart from this dramatic difference, which determines the susceptibility of the host to diarrhea onset, the Ca²⁺-mobilizing properties of both toxins through changes in mucosal cell PLC and phospholipase A2 activity and subsequent mucosal PGE₂ production may, in concert with local ENS reflexes, promote the second phase of diarrhea. If NSP4-induced [Ca²⁺]i mobilization is limited to cells of the intestinal villus and crypt and not found in neuronal plexus cells, an enteroendocrine cell axis for NSP4-ENS interaction should be found.

Finally, sustained [Ca²⁺]i mobilization in cells by a wide variety of agonists leads to cellular stress and a NF-kB-driven cAMP- and PLC-independent diarrhea with both similarities and differences to that evoked by NSP4. This pathway involves the upregulation of mucosal galanin-1 receptors, typically over a time course of 3 days, leading to a Ca²⁺- and pertussis toxin-sensitive fluid (Cl⁻/HCO₃⁻) secretion (19). This innate defense mechanism is “switched on” in mouse colon after enterohemorrhagic E. coli infection and has been reported in vitro after infection with Salmonella and Shigella. Although the cellular mechanism for Cl⁻ secretion is unknown, both enteric nerve- and inflammatory cell-derived galanin sustain this phenomena. Presently, it is unclear whether this represents a pathway common to or separate from that characterized by ischemia-derived reactive hyperemia. Although this Ca²⁺-mediated, CFTR-independent diarrhea resembles that elicited by RV infection and NSP4 inoculation, salient differences include its extended time of onset (days vs. hours for NSP4), the lack of both PLC and age dependence, and the presence of significant mucosal PMN recruitment and/or inflammation. Finally, NSP4 bears no structural resemblance to galanin and would not be expected to activate the galanin-1 receptor. However, the question remains as to whether NSP4 could possibly be circumventing the cellular requirement for this receptor by acting at an intracellular site, for instance, at the level of a common pertussis toxin sensitivity to G protein signaling. In conclusion, although this ENS-dependent mechanism is unlikely to be active during the onset of diarrhea mediated by RV in noninflamed mucosa, an intriguing question remains as to whether NSP4 has evolved to utilize intracellular signaling aspects of the galanin-1 receptor response to promote a second-phase diarrheal disease.

FUTURE DIRECTIONS

There are still many unanswered questions with regard to the cellular basis of the multifaceted RV-induced disease that leads to age-dependent diarrhea. When defining RV secretory diarrhea, current studies have emphasized early events stimulating age-dependent plasmalemmal anion movement at the level of the mucosal cell before more complex secondary phenomena arise. A number of salient questions at this stage of the disease process remain unanswered. At the single-cell level these questions remain: 1) What is the biophysical nature of the NSP4 enterotoxin-stimulated, age- and Ca²⁺-dependent cellular halide secretion? 1) Does non-CFTR-dependent Ca²⁺-activated HCO₃⁻ and/or Cl⁻ transport contribute to the secretory diarrheal response? 3) What is the importance of the non-typical pertussis toxin sensitivity to NSP4-mediated [Ca²⁺]i mobilization, particularly with regard to cellular PLC isoform requirement? 4) What cell type(s) are affected (exocrine, goblet, endocrine)? Even less is known at the whole tissue level and the development of the second-stage diarrheal response. For instance, does a specific cell type transfer NSP4 mucosal signaling effects into the ENS through release of a mucosal cell neuroactive substance, or is this effect more general, reflecting ischemia-derived reactive hyperemia and an innate mucosal defense mechanism? Finally, although RV disease is not characterized by inflammation, how inflammatory conditions within the mucosa affect RV diarrhea remains largely unanswered. The close parallels arising from recent investigations into bacterial microbial interactions with the mucosa and enterotoxi-
generic signaling will provide excellent blueprints for further understanding the pathogenesis of RV- and NSP4-mediated diarrhea.

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